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The Magnetic Immuno Polymerase Chain Reaction Assay for Direct Detection of Salmonellae in Fecal Samples

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Direct polymerase chain reaction (PCR)-based detection with fecal specimens is hampered by inhibitory compounds, such as bilirubin and bile salts. These fecal compounds showed significant inhibition of PCR at low concentrations (10 to 50 μg/ml). For direct PCR analysis, fecal samples must be diluted 500-fold to overcome inhibition. Therefore, the magnetic immuno PCR assay (MIPA), which combines immunomagnetic separation by using specific monoclonal antibodies and PCR, was used to directly detect salmonellae in feces from humans. Immunomagnetically extracted stool samples needed to be diluted only 10-fold when 1 μg of T4 gene 32 protein was added to the PCR. The MIPA sensitivity obtained was 10^5 CFU/ml of feces. A panel of monoclonal antibodies specific for *Salmonella* serogroups A to E was used to extract salmonellae from clinical samples. MIPA detection of salmonellae occurred with 11 out of 14 stool samples stored at 4°C for 2 months. MIPA detection of salmonellae in stool samples is a promising, fast method for detection and identification.

Rapid and accurate diagnostic tests for salmonellae are needed by both the food industry and clinical laboratories. The official culture methods (*Bacterial Analytical Manual*/ Association of Official Analytical Chemists) for the detection of salmonellae in food products require at least 5 days before a negative result can be obtained, and clinical laboratories require at least 2 days to report a definitive positive result from a stool specimen (2, 31). Although progress has been made and a number of practical, rapid *Salmonella* detection methods have been developed, these methods have problems with sensitivity and specificity; direct application to the clinical specimen is often not possible, and enrichment is still required (3, 12, 27).

The polymerase chain reaction (PCR) is a sensitive and rapid technique: a few copies of target DNA can be amplified to a level detectable by gel electrophoresis or hybridization (26). However, PCR-based diagnostics directly from clinical samples have not yet been customized because PCR is sensitive to inhibition by factors present in the crude clinical sample. Blood, urine, and fecal materials are some of the body fluids that inhibit amplification by PCR (11, 14, 33). Inhibition of PCR by body fluids has not been the subject of a systematic study thus far; hemoglobin (16), chelating agents, and other substances that, in general, are capable of inhibiting enzymes have been mentioned. PCR is reportedly inhibited by heparin-collected blood (4), and an unidentified PCR inhibitor appears to copurify with DNA recovered from blood specimens (9). Most PCR-based tests rely on DNA purification by proteinase K, phenol-chloroform extraction, and/or ethanol precipitation (10, 13); examples of alternative methods are extraction with glass beads (7) or silica particles (5) and ion-exchange chromatography (23). However, DNA extraction methods are labor intensive and sensitive to contamination. For routine diagnostics, a simpler method which is amenable to automation is preferred. Immunomagnetic separation (IMS) is one such simple but powerful tool to extract bacteria from samples (21, 22, 29). Bacteria are specifically separated from the specimen, resulting in a useful sample for PCR with little or no nonspecific DNA or interfering factors. The magnetic immuno PCR assay (MIPA) combines specific extraction of bacteria by specific monoclonal antibodies (MAbs) with primer-specific PCR amplification. Recently, we described MIPA for the detection of salmonellae in mixed cultures (32). For PCR amplification, the origin of DNA replication was chosen as the target DNA region (34).

In this paper, the application of MIPA to the detection of salmonellae in stool samples is described. IMS was performed with serogroup-specific MAbs directed against serogroup A to E strains (30). More than 95% of the strains causing infections in humans belong to the serogroups A to E (25). Studies to elucidate the nature of the inhibitors of the PCR in fecal specimens were also performed.

MATERIALS AND METHODS

Bacterial strains and fecal samples. Bacterial strains were obtained from the University Hospital Utrecht and the National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands). The number of CFU was determined by plating on blood agar plates for 16 h at 37°C. Fecal samples were examined for the presence of salmonellae by culturing on salmonella-shigella and Hektoen enteric agar and by enrichment in selenite-cystine broth. The fecal specimens, which were used for detecting *Salmonella typhimurium* in spiked samples, were from patients from the University Hospital Utrecht and were culture negative for salmonellae. Other stool samples were collected during the period 1 to 15 September 1991 at the Academic Hospital Sevilla, Sevilla, Spain; 0.1 to 2 g of feces was diluted in 20 ml of cysteine-peptone transport medium and stored at 4°C for 2 months.

MAbs. *Salmonella* serogroup-specific MAbs were raised and belonged to the immunoglobulin G (IgG) or IgM subclass (30). The following six MAbs were used in this study: MAb

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47.15.1H (IgM), specific for *Salmonella* serogroup A; MAb 71.40.A1 (IgG1), specific for *Salmonella* serogroup B; MAb 72.4.7A (IgG3), specific for *Salmonella* serogroup C1; MAb 35.71.A6 (IgG1), specific for *Salmonella* serogroup C2; MAb 41.11.A3 (IgG1), specific for *Salmonella* serogroup D; and MAb 42.77.A8 (IgG3), specific for *Salmonella* serogroup E. Antibody solutions used for the IMS were hybridoma culture supernatants.

IMS. The assays were conducted with 96-well microtitration plates at room temperature. Dilutions and washing steps were performed with saline.

(i) Detection of *S. typhimurium* in spiked samples. A total 50-μl volume of hybridoma culture supernatant of MAb 71.40.A1 specific for *Salmonella* serogroup B was incubated with 50 μl of 4% (vol/vol) Magnisort M magnetic chromium dioxide particles coated with goat Igs specific for murine IgG and IgM (DuPont, Wilmington, Del.) for 15 min with continuous shaking. The magnetic beads were recovered by magnetic force (MPC-96; Dynal, Oslo, Norway). Stool specimens were inoculated 1:1 with CFU of *S. typhimurium* that ranged from 10^4 to 10^7/ml. After being thoroughly vortexed, the samples were allowed to settle, the supernatant was diluted 10-fold, and 100 μl was used for IMS. After the sample was incubated for 15 min with the magnetic beads with continuous shaking, the magnetic particles were recovered by magnetic force, washed three times, and resuspended in 50 μl of distilled water. The sample was then incubated for 5 min at 100°C and briefly centrifuged, and a 5-μl aliquot from the supernatant was subjected to PCR.

(ii) Detection of salmonellae in patient samples. To prevent competition of the different MAbs for the goat Ig-binding sites, resulting in a lower rate of recovery of the specific bacteria (data not shown), the different MAbs specific for serogroups A to E were incubated separately with the magnetic beads. A 12.5-μl volume of each antibody solution was incubated separately with 12.5 μl of a 4% (vol/vol) magnetic-bead suspension and incubated for 15 min with continuous shaking. The different suspensions were combined, and the magnetic particles were recovered by magnetic force. Stool specimens were diluted 20-fold, thoroughly vortexed, and allowed to settle. Then, 100 μl of the supernatant was used directly for IMS. Further processing of samples was as described above.

Amplification by PCR. Primers used to generate a 163-bp PCR fragment specific for salmonellae were as described previously (32). PCR was performed with *Salmonella* dilutions, diluted stool specimens spiked with *S. typhimurium*, and IMS samples. The PCR mixture (50 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 100 μM (each) deoxynucleoside triphosphate, 0.5 μM (each) primer, 1.0 U of Ampli-Taq polymerase (Cetus, Emeryville, Calif.), and, when indicated, 1 μg of T4 gene 32 protein (Boehringer, Mannheim, Germany). The mixture was then covered with 50 μl of mineral oil. Amplification was conducted with a Perkin-Elmer Thermocycler (Perkin-Elmer Instruments, Norwalk, Conn.) for 35 cycles and followed by a final 10-min extension at 72°C. One cycle consisted of a denaturation step at 94°C for 1 min, a primer-annealing step at 58°C for 1 min, and an extension step at 72°C for 1 s. After being amplified, 20 μl of the sample was electrophoresed on a 1.5% agarose gel. The amplified DNA was visualized by ethidium bromide staining and photographed under UV light. *Pst*I-digested lambda DNA (Boehringer) was used as the size marker. Other thermostable DNA polymerases used were Tth polymerase from *Thermus thermophilus* HB8 (USB Corporation, Cleveland, Ohio) and replinase from *Thermus flavus* (DuPont).

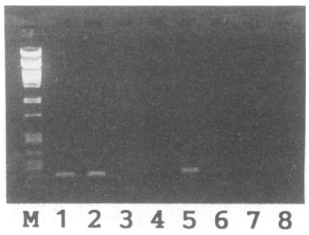

FIG. 1. Inhibition of PCR by bilirubin and bile salts with 1,000 CFU of *S. typhimurium* being used as the PCR template. Lanes: M, molecular weight markers (λ *Pst*I digests); 1, positive control; 2 to 4, 5, 10, and 20 μg of bilirubin per ml; 5 to 7, 20, 50, and 100 μg of bile salts (sodium glycocholate and sodium taurocholate) per ml; 8, negative control.

RESULTS

PCR inhibitory compounds in feces. Undiluted and 10-, 100-, and 500-fold-diluted stool samples, equivalent to 5, 0.5, 0.05, and 0.01 μl of feces, were spiked with 1,000 CFU of *S. typhimurium* and directly analyzed by PCR, and their amplification signals were compared with amplification signals in the absence of feces. Amplification by *Taq* polymerase was significantly hampered by factors in the (diluted) stool suspensions. A 500-fold dilution (equivalent to 0.01 μl of undiluted feces) was required to restore PCR amplification. Hemoglobin degradation products are reported to be the most powerful inhibitory substances found in blood (16). Bilirubin is the principal waste product resulting from the degradation of heme. Therefore, the inhibitory effect of bilirubin was tested with PCR. A standard PCR, containing 1,000 CFU of *S. typhimurium* as the template, was inhibited by a low level of 10 μg of bilirubin per ml (Fig. 1).

In order to obtain more information about the inhibitory compounds of feces, gel filtration was performed. Insoluble particulate matter was removed by brief centrifugation, and the supernatant was filtered through a 0.4-μm-pore-size filter and applied to a Superose gel filtration column (Pharmacia). Aliquots of the eluted fractions, equivalent to 0.1 μl of undiluted feces, were added to the standard PCR. Inhibition of the PCR was proportional to the amounts of protein present in the fractions, indicating that inhibitory factors might be present in the protein-containing fractions. Bile salts are known to possess strong capacities for binding to fecal proteins. Therefore, we investigated the inhibitory effects of the bile salts most abundantly present in feces: sodium glycocholate and sodium taurocholate. As little as 50 μg of these compounds per ml was able to inhibit PCR (Fig. 1).

MIPA detection of *S. typhimurium* in spiked fecal samples. To reduce PCR inhibition of fecal compounds, IMS was performed prior to PCR amplification. T4 gene 32 protein, a single-stranded DNA binding protein, is reported to increase the accessibility of the template DNA to the DNA polymerase in blood samples (24); therefore, we investigated the effect of adding this protein to a PCR of IMS-extracted fecal samples. Amplification enhancement by T4 gene 32 protein is concentration dependent and specific: the strongest enhancement was observed with 1 μg of T4 gene 32 protein, and no effect was observed in the control experiment using 1 μg of ovalbumin (Fig. 2).

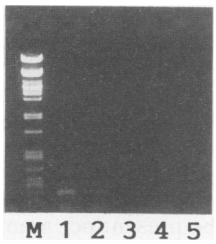

FIG. 2. T4 gene 32 enhancement of PCR with immunomagnetically extracted fecal samples. PCR was performed with 0.5 μl of IMS-extracted feces (10^7 CFU of *S. typhimurium* per ml). Lanes: M, molecular weight markers (λ *Pst*I digests); 1 to 4, 1, 0.3, 0.1, and 0 μg of T4 gene 32 protein; 5, 1 μg of ovalbumin.

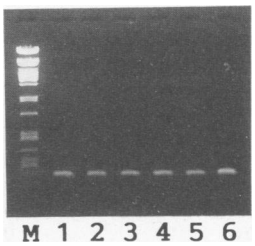

FIG. 4. MIPA with IMS with a panel of MAbs. Lanes: M, molecular weight markers (λ *Pst*I digests); 1 to 6, *S. durazzo* (serogroup A), *S. typhimurium* (serogroup B), *S. virchow* (serogroup C1), *S. newport* (serogroup C2), *S. enteritidis* (serogroup D), and *S. meleagridis* (serogroup E), respectively.

IMS used to preenrich salmonellae in feces before PCR was tested with undiluted and 10-, 25-, and 100-fold-diluted stool samples spiked with 1,000 CFU of *S. typhimurium*. Amplification was not inhibited by the presence of 0.2 μl of IMS-pretreated feces (feces diluted 25×). When 1 μg of T4 gene 32 protein was added to the PCR, 0.5 μl of IMS-pretreated feces (feces diluted 10×) did not interfere with PCR amplification.

Twenty stool samples were tested with 0.5 μl of IMS-pretreated fecal samples with the addition of 1 μg of T4 gene 32 protein for PCR amplification. Since none of the samples were inhibitory to PCR, this protocol was used for further studies. Comparison of Ampli-Taq polymerase with Tth polymerase from *T. thermophilus* HB8 and replinase from *T. flavus* revealed that the latter two enzymes were more sensitive to the inhibitors present in feces (data not shown). The detection limit obtained by MIPA with spiked samples is 10^5 CFU of salmonellae per ml of feces with 0.5 μl of IMS-pretreated feces used as the PCR template (Fig. 3A), whereas 0.5 μl of untreated feces did not yield any positive signal (Fig. 3B). Even when 0.025 μl of feces (diluted 200×) was used as the PCR template, direct PCR was still hampered by inhibitory compounds, with 10^7 CFU/ml obtaining only a faintly detectable signal (Fig. 3C). Larger volumes for IMS, for example, 1 ml instead of 0.1 ml of a 20-fold-diluted sample, and/or the addition of larger volumes of IMS-treated samples to PCR did not increase the sensitivity. We found that the addition of 5% (wt/vol) Chelex 100 (Bio-Rad, Richmond, Calif.) (28), a chelating agent, during the boiling step with IMS-pretreated feces or *Salmonella* suspensions enhanced the amplification signal two- to fourfold, which increased the sensitivity to 2.5×10^4 to 5×10^4 CFU of feces per ml (data not shown).

MIPA with IMS with a panel of MAbs. A panel of MAbs specific for *Salmonella* serogroups A to E was used to separate salmonellae from the test sample. The ability of the panel of MAbs to separate the different salmonellae prior to PCR amplification is shown in Fig. 4. The amount of beads, each coupled with specific MAbs, could be reduced fourfold without a loss of sensitivity compared with that with IMS for *S. typhimurium* in spiked samples.

The results for the application of MIPA to the detection of salmonellae in feces stored in transport medium at 4°C for 2 months are shown in Table 1. Eleven out of 14 fecal specimens were positive. All 14 samples were culture positive after enrichment; isolated *Salmonella* strains were identified as *S. enteritidis*, *S. typhimurium*, and *S. montevideo*. The MIPA-negative samples, which contained fewer than 10^4 CFU of salmonellae per ml, were all MIPA positive after enrichment.

DISCUSSION

Direct PCR with fecal samples is hampered by the inhibitory compounds present in feces. Studies to elucidate the nature of these inhibitory compounds were conducted. Bilirubin, a degradation product of hemoglobin, is inhibitory at the low concentration of 10 μg/ml. It is reduced by bacteria in the gut to urobilinogens, and its excretion by healthy persons can reach 350 mg/day; patients may have even higher levels (8). Gel filtration of diluted feces has indicated that inhibitory compounds are present in protein-containing fractions, suggesting that bile salts, bound to fecal proteins, may be inhibitory. The excretion of bile salts by normal adults reaches 200 to 650 mg/day; a 10-fold increase may be observed with patients with ileal dysfunctions (15). PCR is inhibited by the presence of 50 μg of bile salts per ml. The observed PCR inhibitory levels for bile salts and bilirubin-urobilinogens can be reached with slightly diluted or undiluted feces. Thus, direct PCR analysis of clinical specimens

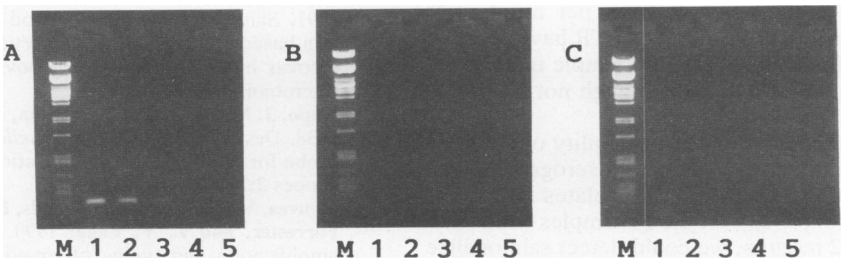

FIG. 3. Comparison of MIPA with direct PCR. PCR was conducted with the addition of 1 U of T4 gene 32 protein. Results of PCR with 0.5 μl of IMS-extracted feces (A) and direct PCR with 0.5 (B) and 0.025 (C) μl of feces are shown. Lanes: M, molecular weight markers (λ *Pst*I digests); 1 to 5, 10^7, 10^6, 10^5, 10^4, and 0 CFU of *S. typhimurium* per ml of feces.

TABLE 1. MIPA for clinical stool specimens with IMS with a panel of MAbs against *Salmonella* serogroups A to E

Isolate	Serotype	Amt as PCR template (μl of IMS-treated feces)[a]	Result of MIPA[b]
S23	*S. montevideo*	0.3	+
S63	*S. montevideo*	0.1	+
S66	*S. enteritidis*	0.05	+
S79	*S. enteritidis*	0.35	+
S89	*Salmonella* serogroup B (untypeable)	0.35	−
S90	*S. enteritidis*	0.35	−
S96	*S. enteritidis*	0.5	−
S119	*S. enteritidis*	0.4	+
T38	*S. typhimurium*	0.35	+
T40	*S. enteritidis*	0.5	+
T42	*S. enteritidis*	0.5	+
T43	*S. enteritidis*	0.5	+
T44	*S. typhimurium*	0.5	+
T49	*S. enteritidis*	0.5	+

[a] Samples were, if possible, diluted 20-fold, resulting in a PCR template of 0.5 μl of feces. Some samples, however, were diluted more than 20-fold in the transport medium, resulting in a smaller amount of feces as the PCR template.
[b] +, positive; −, negative.

is possible only after extensive dilution. This, however, makes the assay less sensitive, since fewer bacteria are present in the diluted sample (1, 33). When IMS was used to preenrich salmonellae, the dilution of feces needed to overcome inhibition was 20-fold lower than that needed for direct PCR with feces. The addition of 1 μg of T4 gene 32 protein, a single-stranded DNA binding protein, enhanced PCR amplification of the target DNA in feces. Thus, IMS-pretreated stool samples with the addition of T4 gene 32 protein needed to be diluted only 10-fold, which is equivalent to 0.5 μl of IMS-pretreated feces as the PCR template.

MIPA for the detection of *Escherichia coli* heat-stable enterotoxin in pig stool specimens has already been described (17); even though IMS was used for preenrichment, problems with the necessary dilutions of feces were still encountered. Using IMS and T4 gene 32 protein, we were able to use 0.5 μl of IMS-pretreated human feces as the standard PCR template without having problems with the dilution rates. Since only a 10-fold dilution of feces was used, the rate of occurrence of false-negative results is probably low for patients with clinical symptoms. The sensitivity of MIPA was 10^5 CFU of feces per ml. This corresponds to 50 CFU per PCR, and it is similar to the sensitivity obtained with bacterial suspensions (32). Recent studies have indicated that the addition of Chelex 100, a chelating agent, during the boiling step with IMS-pretreated feces enhanced amplification and that the obtained detection limit was 2.5×10^4 to 5×10^4 CFU of feces per ml. Larger volumes of feces for IMS pretreatment or PCR have resulted in larger amounts of inhibitors. IMS is a simple method for significantly reducing inhibitors, even though not all inhibitors can be removed.

In this study, we demonstrated the possibility of a pooled MAb-bead suspension to cover *Salmonella* serogroups A to E in IMS. More than 95% of the clinical isolates in humans belong to these serogroups (25). Of the 14 samples which had been refrigerated for 2 months, we could detect salmonellae in 11. The most frequently isolated strain was *S. enteritidis*, followed by *S. typhimurium* and *S. montevideo*. MIPA-negative samples contained too few salmonellae for positive identification. MIPA conducted with enrichment broths of the negative samples became positive, indicating that negative results were not caused by MAbs or by primer nonspecificity. It has been found that storage and transport in transport medium dramatically decreases the numbers of live salmonellae (19, 20). Therefore, the sensitivity of MIPA is sufficient for application on fresh stool samples, since during gastroenteritis as many as 5×10^5 to 5×10^9 CFU of salmonellae may be present per gram of feces (6, 18).

The described combination of IMS, with a pooled MAb-bead suspension used to cover *Salmonella* serogroups A to E, and PCR seems to be a suitable method for rapid detection of salmonellae in fresh fecal specimens from humans. MIPA is not restricted to *Salmonella* spp. and can be expanded to other bacteria or viruses, depending on the specific antibodies and primer sets used.

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